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Reversal of Experimental Autoimmune Encephalomyelitis by a Soluble Peptide Variant of a Myelin Basic Protein Epitope: T Cell Receptor Antagonism and Reduction of Interferon γ and Tumor Necrosis Factor α Production

By Nathan Karin, Dennis J. Mitchell, Stefan Brocke, Nicholas Ling,* and Lawrence Steinman

*From the Department of Neurology and Neurological Sciences, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, California 94305-5239; and *Neurocrine Biosciences, Inc., La Jolla, CA 92121*

Summary

An immunodominant epitope of myelin basic protein (MBP), VHFFKNIVTPRTP (p87–99), is a major target of T cells in lesions of multiple sclerosis (MS) and in experimental allergic encephalomyelitis (EAE). T cells found in EAE lesions bear the same amino acids in the third complementary determining region of the T cell receptor (TCR) as those found in MS lesions. We analyzed the trimolecular interactions between MBP p87–99, class II major histocompatibility complex (MHC), and TCR, and designed soluble inhibitors for therapy. F, N, I, and V at positions 90, 92, 93, and 94 interact with MHC, whereas K, T, and P at positions 91, 95, and 96 interact with TCR. The peptides, p87–99[95T>A] and p87–99[96P>A] could compete more effectively with p87–99 for binding to MHC and could antagonize the in vitro response of T cells to p87–99 more effectively than p87–99[91K>A]. However, only p87–99[91K>A] prevented and reversed EAE, indicating that the extent of MHC or TCR competition does not predict success in treating EAE. To elucidate the mechanism of inhibition of EAE, draining lymph node cells from rats immunized with the native peptide alone or together with each of the three TCR antagonists were challenged in vitro with p87–99. Administration of p87–99[91K>A], but not p87–99 [95T>A] or p87–99[96P>A], reduced the production of tumor necrosis factor (TNF)- α and interferon (IFN) γ. IFN-γ and TNF-α are two cytokines that are critical in the pathogenesis of EAE and MS.

Experimental allergic encephalomyelitis (EAE)[1] is a T cell–mediated autoimmune disease of the central nervous system (1, 2). The TCR recognizes peptide bound to an MHC molecule (3). Several groups, including ours, have formulated nonimmunogenic peptides based on the sequence of myelin basic protein (MBP) that bind class II MHC to a much greater extent than the native, encephalitogenic peptides of MBP, and that prevent EAE when given in adjuvant (4–9). We now extend this approach in order to develop TCR antagonists that would prevent and reverse EAE, when induced by MBP p87–99.

The rationale for pursuing inhibitors of the MBP p87–99 follows from some serendipitous findings (10) regarding a major set of TCR rearrangements in multiple sclerosis (MS) brain lesions. Using PCR on reverse-transcribed mRNA, we analyzed TCR gene rearrangements directly from brain plaques of MS patients, who are HLA DR2 (10), and from spinal cords of rats with EAE after immunization with p87–99 (11). T cells with the amino acid motif LRG in the CDR3 region (10–13) are found in EAE lesions in the Lewis rat and in MS lesions. Remarkably, a cytotoxic T cell clone specific for MBP p87–99 from an MS patient who was HLA DR2 has the same amino acid motif, LRG, in the CDR3 of its TCR-β chain (12, 14). Thus, T cell clones in Lewis rats and in MS patients, which express this CDR3 motif, all have specificity for MBP p87–99, VHFFKNIVTPRTP (p87–99) (10, 11, 14). MBP p87–99 has further importance because it is an immunodominant epitope of MBP in MS patients who are HLA DR2 (10, 11, 14, 15).

We have analyzed the putative sites where MBP p87–99 interacts with MHC and TCR in the Lewis rat. Based on these studies, we designed different peptide antagonists of MBP 87–99 that interfere with the encephalitogenic T cells. We have been able to design a peptide inhibitor that an-

[1] *Abbreviations used in this paper:* EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; PLP, proteolipid protein; SI, stimulation index.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/12/2227/11 $2.00
Volume 180 December 1994 2227–2237

tagonizes the pathogenic T cells and when administered in soluble form, can reverse ongoing EAE with clinical paralysis. This peptide also reduces production of two critical cytokines involved in the pathogenesis of EAE and MS, TNF-α and IFN-γ. This report extends the approach of using TCR antagonists to prevent EAE (4, 5, 8, 16). Recently, Franco et al. (16) showed that they could prevent EAE with a pool of TCR antagonist peptides given in equimolar amounts with the encephalitogenic peptide 139–151 of proteolipid protein (PLP) in CFA. We sought to apply this approach to a clinically relevant paradigm in which we attempted to reverse established paralysis with a TCR antagonist that could be given in a soluble form.

Materials and Methods

Rats. Female Lewis rats, ~6 wk old, were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Peptide Antigens. Human MBP p87–99, VHFFKNIVTPRTP, and a series of 13 p87–99 analogues that differ from the original MBP peptide by a single alanine substitution, as well as MBP p1–20 and MBP p68–86, were synthesized with a free carboxylic acid at the COOH terminus on a peptide synthesizer (model 9050; MilliGen, Burlington, MA) by standard 9-fluorenylmethoxycarbonyl chemistry. MBP p1-20 is acetylated at the NH_2 terminus. Peptides were purified by HPLC. Structure was confirmed by amino acid analysis and mass spectroscopy. Only peptides that were >95% pure were used in our study.

Immunizations and Active EAE Induction. MBP peptides were dissolved in PBS and emulsified with an equal volume of IFA supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra in oil (Difco Laboratories, Detroit, MI). Rats were immunized subcutaneously in the hind foot pads with 0.1 ml of the emulsion and were monitored daily for clinical signs by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; and 3, front and hind limb paralysis.

Culture Media. DME (GIBCO BRL, Gaithersburg, MD) supplemented with 2-ME (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 μ/ml), streptomycin (100 μg/ml), and 1% autologous Lewis rat serum was used as a stimulation medium. Resting medium was identical to the stimulation medium without autologous serum and with the addition of 10% FCS (GIBCO BRL) and 12.5% supernatant of Con A–stimulated splenocytes as a source of T cell growth factors. Con A supernatant was prepared as described elsewhere (1).

Antigen-specific T Cell Proliferation Assays. Lewis rats were immunized with different MBP peptides as described above. 9–10 d later, draining lymph node cells were suspended in stimulation medium and cultured in U-shape 96-well microculture plates for 72 h at 37°C in humidified air containing 6.5% CO_2, at a concentration of 2×10^5 cells/well. For the antigen-specific proliferation assay of T cell lines and clones, cells were plated at a concentration of 2×10^4 cells/well with 10^6 irradiated (2,500 rad) thymocytes as accessory cells together with different concentrations of antigen. Each well was pulsed with 2 μCi of [^{3}H]thymidine (sp act 10 Ci/mmol) for the final 6 h. The cultures were then harvested on fiberglass filters and the proliferative response expressed as cpm ± SD or as stimulation index (SI) (mean counts per minute test cultures divided by mean counts per minute control cultures).

Long-term T Cell Lines and Clones. Antigen-specific long-term T cell lines and clones were derived by use of the method developed by Ben-Nun et al. (1). Lewis rats were immunized as described previously. 9–10 d later, draining lymph node cells were cultured (10^7/ml) for 72 h in stimulation medium together with 10–20 μmol of the immunizing peptide. The cells were then collected, washed, and cultured for a phase in resting medium. After an additional 5–8 d, cells were collected and either tested for antigen-specific proliferation or cultured for additional cycles. For cloning of antigen-specific T cell lines, resting T cells were cultured in 96-well microculture plates at a concentration of 0.3 cells/well with 5×10^5 irradiated (2,500 rad) thymocytes as accessory cells and 10–20 μmol of antigen. After three to five stimulation cycles, plates were screened, clones were transferred to 24-well flat bottom plates, and restimulated. Before use in the study, all clones were recloned by use of the method described above.

MHC Binding Assay. An assay that characterizes the binding of peptides to MHC molecules on living APCs was used (17, 18). Spleen cells were cultured in DME supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) in standard polystyrene petri dishes (100 × 15 mm; Baxter Healthcare Corp., Deerfield, IL) in a 37°C incubator containing 6.5% CO_2 for 3 h. Thereafter, nonadherent cells were removed, and the plates were washed three times with PBS. Adherent cells were collected with a cell scraper (Falcon, Franklin Lakes, NJ). The binding of p87–99 analogues was measured by use of a fluorescence assay as follows: 5×10^5 splenic adherent cells in staining buffer containing 0.1% BSA in PBS were mixed with different concentrations of p87–99 analogues in individual wells of U-shape 96-well microculture plates and incubated at 37°C in a 6.5% CO_2 incubator. 1 h later, biotin-labeled p87–99 (10 μmol) was added to culture wells for 4 h. The cells were washed three times with the staining buffer before PE–streptavidin (Becton Dickinson & Co., San Jose, CA) was added as a second-step reagent (10 μl/well, 20 min) along with labeled mAb reacting with rat MHC class II I-A (0.4 μl/well, OX-6; PharMingen, San Diego, CA). The cells were washed twice before cytofluorographic analysis on a FACScan® (Becton Dickinson & Co.). The fluorescence intensity was calculated by subtracting the fluorescence obtained from OX-6–positive cells stained with PE–streptavidin only from the fluorescence obtained with biotin-labeled p87–99 plus phycoerythrin–streptavidin. The percentage of inhibition was calculated and is presented as IC_{50} values.

T Cell Antagonism Proliferation Assay. T cell antagonism was detected in a prepulsed proliferation assay as described by De Magistris et al. (19) with some modifications. Antigen-presenting spleen cells were γ irradiated (3,000 rad) and incubated with 0.2 μM of the native peptide in stimulation medium at a concentration of 10^7 cells/well in 10-ml tissue culture plates for 2.5 h at 37°C in humidified air containing 6.5% CO_2. Spleen cells were then washed and recultured in individual wells of U-shape 96-well microculture plates (5×10^5 cells/well) with 5×10^4 resting anti-p87–99 T cells with different concentrations of antagonist peptides, ranging from 10^{-4} μM to 10^{-1} μM, for an additional 60 h. Each well was pulsed with 1 μCi of [^{3}H]thymidine (sp act 10 Ci/mmol) for the final 18 h. The cultures were then harvested on fiberglass filters and the proliferative response expressed as cpm ± SD or SI (mean cpm test cultures divided by mean cpm control cultures).

Cytokine Determination. Draining lymph node cells were stimulated in vitro (10^7 cells/ml) with different concentrations of antigens. After 12, 24, 48, and 72 h of stimulation, supernatants were collected for cytokine detection, and cells were immediately frozen for RNA extraction. IFN-γ was determined after 48 h by use of a rat IFN-γ ELISA kit (GIBCO BRL). TNF-α was measured after 24 h by use of ELISA kits (Genzyme Corp., Cambridge, MA) suitable for mice and rats. The kits were used according to manufacturers' instructions.

Results

Determination of Amino Acids Involved in the Binding of p87–99 to RT.D¹ (I-E) MHC Class II Molecules. A single injection of guinea pig MBP emulsified in CFA induces EAE in the Lewis rat (1). The T cell response is directed against a major encephalitogenic region encompassed by residues 68–86 presented in association with RT.B¹ (I-A) MHC class II molecules (20), and to a second encephalitogenic region encompassed by a residues 87–99 of MBP restricted by RT.D¹ (I-E) MHC class II gene products (20, 21). A T cell line specific for p87–99, named L87–99, was selected. The proliferative response of the line was blocked to a substantial degree with an anti-I-E (OX-17, 2.5 μg/ml), but not an anti-I-A (OX-6, 2.5 μg/ml) mAb (response to MBP p87–99: 54,350 ± 840 with anti-I-A blocking vs. 17,120 ± 310 with anti-I-E blocking [data shown as cpm ± SE, background: 3,420 ± 330 cpm, $p < 0.001$]). A panel of 13 alanine-substituted peptides based on MBP p87–99 was synthesized (Table 1). These analogues were tested in a competition assay with the native peptide for binding to I-E on APCs (Fig. 1, Table 1). Four of these peptides, p87–99[90F>A], p87–99[92N>A], p87–99 [93I>A], and p87–99[94V>A], were poor competitors with the native peptide on binding to autologous APCs (IC_{50} >200 μM for each of the analogues, compared with IC_{50} = 14 μM for the native peptide) (Table 1). These results indicate that these four positions are putative binding sites to I-E (see Fig. 3).

Amino Acids Necessary for Induction of EAE. Each of 13 alanine-substituted analogues was tested for its ability to induce EAE (Table 1). Immunization with the native peptide, as well as 6 of the other 13 mutated peptides, p87–99[87V>A], p87–99[88H>A], p87–99[89F>A], p87–99[93I>A], p87–99 [97R>A], and p87–99[98T>A], elicited EAE in 34 of 36 Lewis rats. In contrast, the three peptide analogues, p87–99 [91K>A], p87–99[95T>A], and p87–99[96P>A], with an IC_{50} for binding to I-E that was similar to native peptide (Table 1, Fig. 1), (range of IC_{50} 14–21 compared with IC_{50} native peptide of 14), never induced EAE in Lewis rats (Table 1, incidence of 0.6 for each of these analogues compared with 6/6 for the native peptide). This suggests that lysine, threonine, and proline at positions 91, 95, and 96 are essential for TCR recognition of p87–99.

All three nonencephalitogenic peptide analogues that bound to I-E with IC_{50} in the range of native peptide, p87–99 [91K>A], p87–99[95T>A], and p87–99[96P>A], either stimulated a weak response to p87–99 or failed to elicit a proliferative response in L87–99 (Fig. 2). This further confirms our observation that the lysine, threonine, and proline at positions 91, 95, and 96 are essential for effective TCR binding to p87–99 (Figs. 2 and 3). Consequently, each of these analogues might serve as a therapeutic TCR antagonist capable of blocking EAE. The peptides p87–99[97R>A] and p87–99 [98T>A] were encephalitogenic and could evoke a proliferative response in draining lymph node T cells after immunization with native p87–99 (Table 1), though they failed to elicit a significant proliferative response in L87–99 (Fig. 2). This indicates that L87–99 does not include all potential T cell clones capable of mounting a pathogenic T cell response.

Comparison of the TCR Antagonists p87–99[95T>A], p87–99 [96P>A], and p87–99[91K>A]. Using an assay developed by De Magistris et al. (19) to detect TCR antagonists, we

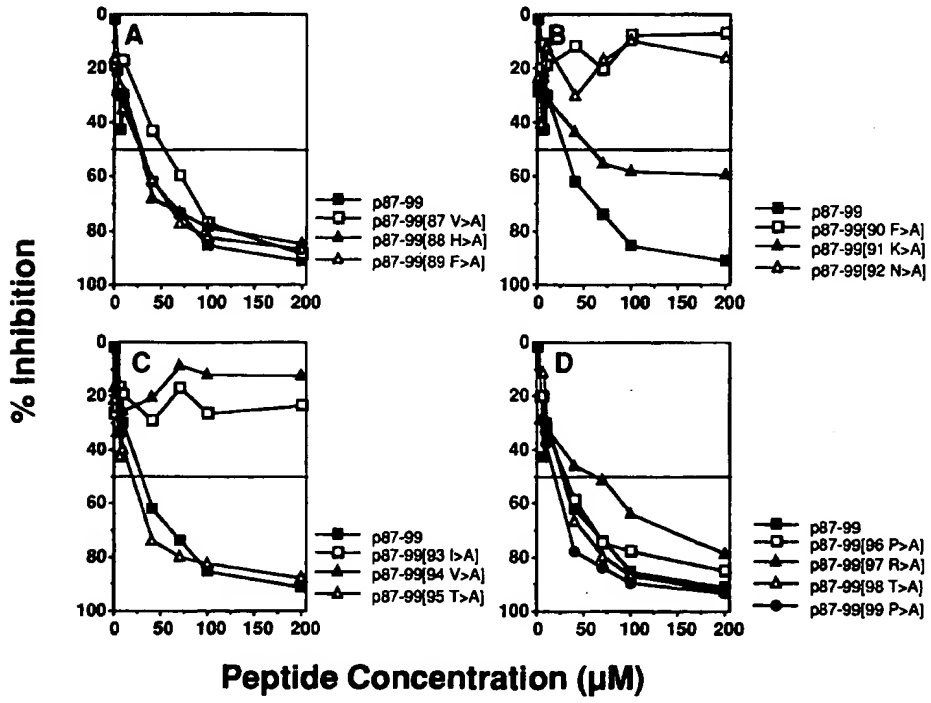

Figure 1. MHC binding competition between alanine analogues and MBP p87–99. The ability of each of the 13 alanine-substituted analogues at different concentrations from 0 to 200 μM to inhibit the binding of 10 μM of biotin-labeled native peptide was measured and presented as the percentage of inhibition of mean relative binding. The IC_{50} values are shown in Table 1.

Table 1. *The Effect of Single Alanine Substitutions on Relative MHC II Binding and Encephalitogeneity of the Peptide*

Peptide	87	88	89	90	91	92	93	94	95	96	97	98	99	IC_{50} I-E binding (μM)*	DLNC proliferative response‡	Line 87-99 proliferative response§	Active EAE Incidence	Active EAE Duration
																		d
p87-99	V	H	F	F	K	N	I	V	T	P	R	T	P	14	+ + + +	+ + + + +	6/6	7
p87-99 [87V>]	A													31	+ + + + +	+ + + +	6/6	7
p87-99 [88H>A]		A												14	+ + + + +	+ + + +	6/6	7
p87-99 [89F>A]			A											11	+ + + +	+ + +	6/6	7
p87-99 [90F>A]				A										>200	+	−	0/6	0
p87-99 [91K>A]					A									21	+	−	0/6	0
p87-99 [92N>A]						A								>200	+	+ + + +	1/6	4
p87-99 [93I>A]							A							>200	−	+ + +	6/6	7
p87-99 [94V>A]								A						>200	−	+ +	2/6	5
p87-99 [95T>A]									A					14	+	−	0/6	0
p87-99 [96P>A]										A				14	+ +	−	0/6	0
p87-99 [97R>A]											A			20	+ +	−	4/6	7
p87-99 [98T>A]												A		14	+ + + + +	+ +	6/6	7
p87-99 [99P>A]													A	10	+ + + + +	+ + + +	3/6	5

* IC_{50} values were calculated from Fig. 1.

‡ Rats were immunized with p87-99. 9 d later, the proliferative response against the native peptide, as well as against each of the 13 peptide analogues, was determined in a proliferation assay. The maximal response was graded as follows: −, SI <1; +, 1 <SI <1.5; + +, 1.5 <SI <2; + + +, 2 <SI <3; + + + +, 3 <SI <3.5; + + + + +, 3.5 <SI <4.

§ The proliferative response of line L87-99 to its native peptide as well as to all the peptide analogues was recorded (Fig. 2). These results are summarized here as follows: −, SI <20; +, 20 <SI <30; + +, 30 <SI <40; + + +, 40 <SI <50; + + + +, 50 <SI <75; + + + + +, 75 <SI <85.

tested p87-99[91K>A], p87-99[95T>A], and p87-99-[96P>A] for their capacity to inhibit the response of L87-99 to native peptide MBP p87-99. A concentration of 20 μM of p87-99 was found to be optimal for induction of a maximal proliferative response in L87-99 (Fig. 2). We tested TCR antagonism at a concentration of 0.2 μM since this concentration elicited a significant response in L87-99 (Fig. 4, SI = 23.0 ± 1.1 with a background of 306 cpm). All three analogues antagonized the response of L87-99 to native peptide when added at a concentration of 0.0001–0.01 μM in the proliferation assay (Fig. 4). In fact, p87-99[95T>A] and p87-99[96P>A] were found to be stronger antagonists than p87-99[91K>A]. At a concentration as low as 0.0001 μM of p87-99[95T>A] or p87-99[96P>A], >85% inhibition of the proliferative response of the encephalitogenic line to its native peptide (SI = 2.6 ± 0.2 and 1.9 ± 0.2 compared with SI = 23 ± 1.1, p <0.001) (Fig. 5, *B* and *C*) was observed. A 100-fold greater concentration of peptide p87-99-[91K>A] was required for a similar degree of inhibition (SI = 4.7 ± 2.7 compared with SI = 23.0 ± 1.1, p <0.001) (Fig. 4 *A*). These findings could be explained in part by the fact that both p87-99[95T>A] and p87-99[96P>A] are stronger competitors of the native peptide on binding I-E than p87-99[91K>A] (IC_{50} = 14 for each of these peptides compared with IC_{50} = 21 for p87-99[91K>A]) (Fig. 1).

All three peptide analogues antagonized the proliferative response of the encephalitogenic line L87-99 (Fig. 4). We also tested each of these antagonists on an anti-p87-99 $CD4^+$ T cell clone selected from this line. A representative experiment with this clone revealed a proliferative response of 12,336 ± 440 cpm with a background of 458 cpm in response to 0.2 μM of p87-99. Each of the putative antagonists inhibited this response. At 0.001 μM of either p87-99 [91K>A], p87-99[95T>A], or p87-99[96P>A], proliferation was reduced to 6,450 ± 530, 2,458 ± 670, and 3,430 ± 175 cpm, respectively.

p87-99[91K>A], but neither p87-99[95T>A] nor p87-99 [96P>A], Prevents EAE in an Epitope-specific Manner. Since p87-99[95T>A] and p87-99[96P>A] displayed stronger in vitro blocking capacities for I-E and TCR than p87-99 [91K>A], they were both expected to inhibit EAE better than p87-99[91K>A]. Interestingly, only p87-99[91K>A] (0/12 with EAE vs. 24/24 groups g and h, p <0.001) could prevent EAE when coimmunized with native peptide at a 1:1 molar ratio (Table 2, group *h* compared with *g* and *f*). Neither p87-99[95T>A] (12/12 with EAE) nor p87-99[96P>A] (12/12 with EAE) could prevent EAE when coimmunized with native peptide at a 1:1 molar ratio (Table 2). p87-99 [91K>A] reduced the incidence and severity of disease even at a 3:1 excess of the pathogenic peptide (3/6 with EAE; Table 2, group *i*).

The NH_2-terminal fragment of MBP (p1–20) was used as

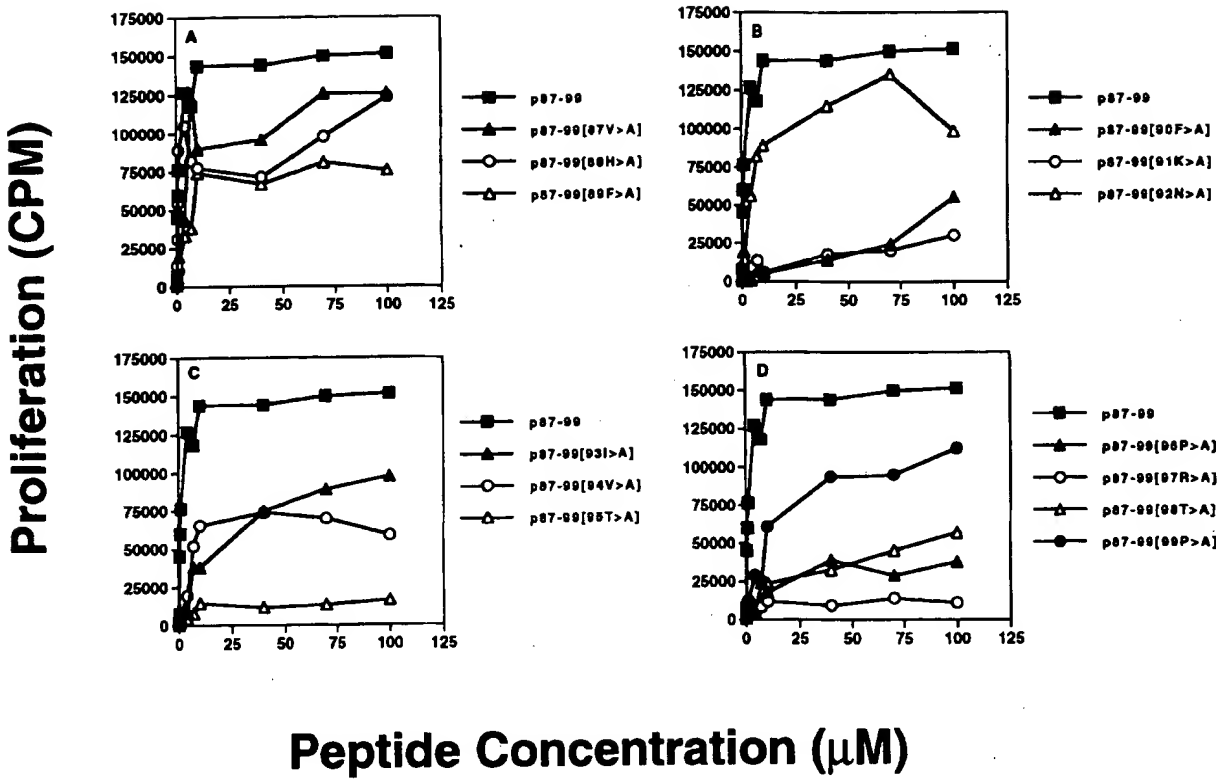

Figure 2. Proliferative response of L87–99 to different alanine-substituted analogues. The proliferative response of L87–99 in response to the different alanine-substituted analogues was determined in a proliferation assay and shown as counts per minute. All standard deviations were less than ±10%.

a control peptide, since in Lewis rats this peptide is not encephalitogenic (Table 2, group *l*). Coimmunization of the p1–20 fragment together with p87–99 did not prevent EAE in any of the immunized rats (6/6 with EAE; Table 2, group *m*).

In Lewis rats there are two encephalitogenic regions of MBP, p68–86 and p87–99. We studied the specificity of EAE inhibition by p87–99[91K>A]. Rats were coimmunized with either p87–99 plus p87–99[91K>A] or p68–86 plus p87–99

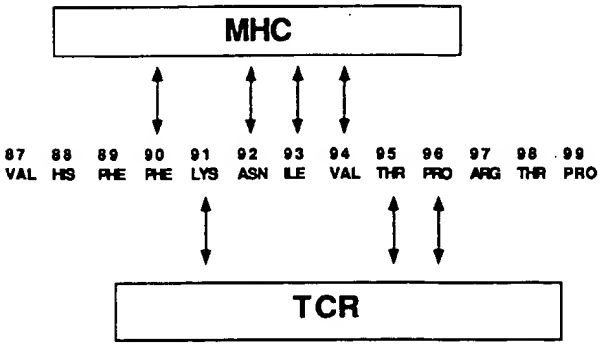

Figure 3. Putative binding site of p87–99 to MHC and TCR. Putative binding sites of p87–99 to MHC and TCR were inferred according to the results summarized in Table 1.

[91K>A] (at a 1:1 molar ratio for each combination), or with each of the native peptides alone. Prevention of EAE by p87–99[91K>A] was specific for disease induced with p87–99 (18/18 with EAE vs. 0/12 with EAE, $p < 0.001$), but not with disease induced with p68–86 (6/6 with EAE; Table 2, groups *a*, *h*, *j*, and *k*). Thus, p87–99[91K>A] prevents EAE in an epitope-specific manner.

Mechanisms of Inhibition of EAE by p87–99[91K>A]. To further investigate the mechanism behind EAE resistance, rats were immunized with native peptide p87–99 alone, or together with either peptide p87–99[91K>A], p87–99[95T>A], or p87–99[96P>A] at a 1:1 molar ratio with the native peptide, p87–99. 9 d later, some of the rats were killed, and proliferative responses against the native peptide were determined in the draining lymph nodes (Fig. 5, *A* and *B*). Some rats from each of the groups remained in cages for further observation for development of paralysis. All rats (6/6) immunized with the native peptide alone or p87–99 together with either p87–99[95T>A] or p87–99[96P>A] developed EAE. None of the rats coimmunized with p87–99[91K>A] and p87–99 developed EAE (0/6, $p < 0.001$). Nevertheless, all groups displayed a very similar SI (Fig. 5 *B*) in the proliferative response against the native peptide, p87–99, in their draining lymph node cells. We noted that the proliferative response to p87–99 (in absolute counts per minute) observed in draining lymph node cells from rats immunized with the

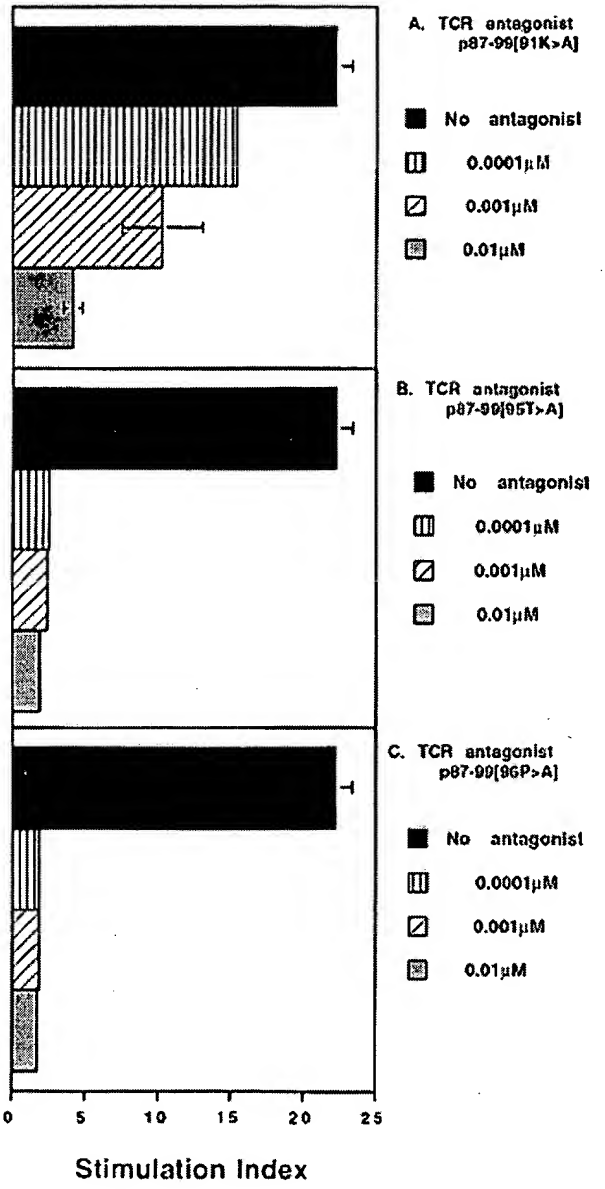

Figure 4. TCR antagonism of alanine-substituted analogues for MBP p87–99. The proliferative response of L87–99 to 0.2 μM of the native peptide was antagonized by 0.0001–0.01 μM of either p87–99[91K>A] (*A*), p87–99[95T>A] (*B*), or p87–99[96P>A] (*C*). Results are shown as SI ± SE.

native peptide alone was lower than the proliferative response seen in lymph nodes from rats coimmunized with p87–99 [91K>A], p87–99[95T>A], or p87–99[96P>A] (Fig. 5, *A* and *B*). These results indicate that EAE resistance did not result from induction of unresponsiveness to the native, pathogenic peptide when rats were coimmunized with native peptide and p87–99[91K>A].

We hypothesized that the protective and nonprotective TCR antagonists might differentially alter the cytokine profile in encephalitogenic T cells after stimulation with the pathogenic peptide. The draining lymph node cells from rats coimmunized with p87–99 and p87–99[91K>A] produced markedly reduced levels of TNF-α and IFN-γ compared with those immunized with the native peptide or coimmunized with p87–99[95T>A] or p87–99[96P>A]. For IFN-γ, we observed the following amounts in response to 100 μM p87–99 in vitro (Fig. 5 *C*): 1,750 ± 80 pg/ml when p87–99[91K>A] was coimmunized with native p87–99, compared with 5,200 ± 300 pg/ml for p87–99[95T>A] plus p87–99 ($p < 0.0015$); 7,200 ± 580 pg /ml for p87–99[96P>A] plus p87–99 ($p < 0.001$); and 4,200 ± 100 pg/ml for native peptide alone ($p < 0.003$). For TNF-α, we observed the following amounts in response to 100 μM p87–99 in vitro (Fig. 5 *D*): 32 ± 3 pg/ml when p87–99[91K>A] was coimmunized with native p87–99, compared with 81 ± 4 pg/ml for p87–99 [95T>A] plus p87–99 ($p < 0.003$); 72 ± 4 pg/ml for p87–99 [96P>A] plus p87–99 ($p < 0.004$); and 62 ± 1 pg/ml when p87–99 was given alone ($p < 0.005$). Thus the TCR antagonist capable of blocking EAE, p87–99[91K>A], reduced TNF-α and IFN-γ production, whereas the other TCR antagonists did not influence the production of these cytokines.

Reversal of EAE by Soluble Peptide Analogues. From a clinical perspective, the use of a soluble form of a therapeutic peptide analogue would be more practical than the use of such a peptide emulsified in CFA. We therefore explored the therapeutic potential of soluble p87–99[91K>A]. In experiment 1 (Table 3), a single i.p. inoculation with 2 mg/ml of either p87–99 or p87–99[91K>A] 2 d after transfer of L87–99 reduced the incidence of EAE (for example, 5/5 control with EAE, day 5, vs. 0/5 with p87–99, vs. 0/5 with p87–99 [91K>A], $p < 0.002$). Neither p87–99[95T>A] nor p87–99 [96P>A] prevented EAE.

In experiment two (Table 3), two i.p. injections of 2 mg/ml each were given on days 2 and 4 after transfer of L87–99. Peptides p87–99, p87–99[91K>A], and p87–99[96P>A] reduced the incidence of EAE: on day 7 in the group of control rats, 6/6 had EAE, but when p87–99 was given intraperitoneally in solution, 0/6 had EAE ($p < 0.001$ compared with control animals), when p87–99[K91>A] was given intraperitoneally in solution, 0/6 had EAE ($p < 0.001$ compared with control rats), and when p87–99[96P>A] was given intraperitoneally in solution, 2/6 had EAE ($p < 0.15$ compared with control rats). In comparison, when p87–99[95T>A] was given intraperitoneally in solution, 6/6 had EAE.

Disease reversal was attempted in a third experiment in which 15 rats were inoculated with 15×10^6 L87–99 cells to induce EAE and were randomly distributed into three subgroups of 5 rats each. 1 d later, two of these groups were given intraperitoneally 2 mg/ml of p87–99[91K>A] dissolved in PBS. All 15 rats had hind limb paralysis 4–5 d after disease induction. On day 5, one group received another 2 mg/ml of p87–99[91K>A]. All 5 rats who received this second infusion of p87–99[91K>A] went into complete remission within 24–36 h, with no residual signs of paralysis, whereas paralysis persisted in all other rats for another 4–5 d.

In a fourth experiment (Fig. 6), 18 rats were given 10^7 L87–99 T cells, and all 18 developed hind limb paralysis within

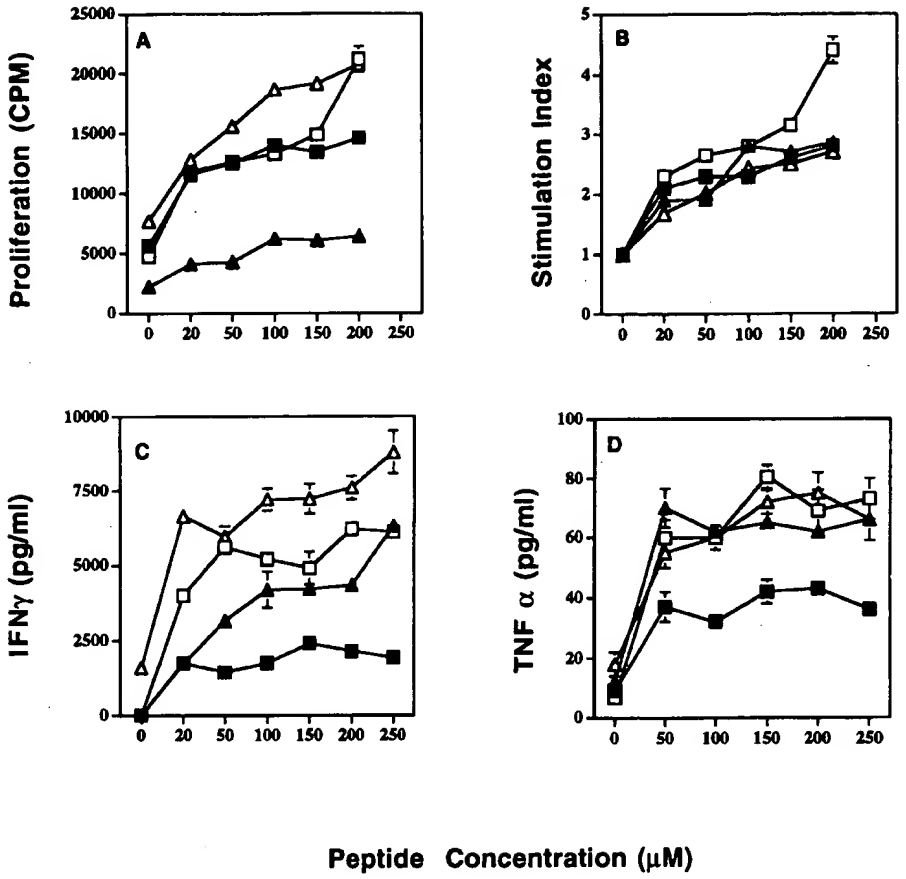

Figure 5. Cytokine production altered by peptide analogues. The proliferative response (*A* and *B*), IFN-γ (*C*), and TNF-α (*D*) production in draining lymph node cells from rats immunized with p87–99 alone (–▲–), or together with p87–99[91K>A] (–■–), p87–99[95T>A] (–□–), or p87–99[96P>A] (–△–). Proliferative responses were analyzed either as counts per minute ± SE (*A*) or by SI (*B*).

5 d. The paralyzed rats were given a single injection (2 mg/ml) of soluble p87–99[91K>A], p87–99[95T>A], or PBS. Although all positive control PBS-immunized rats, as well as those immunized with p87–99[95T>A] (6/6 in each group), continued to show hind limb paralysis for the following 4 d, all rats (6/6) treated with the p87–99[91K>A] analogue went into complete remission within 36 h without further signs of paralysis ($p < 0.015$) (Fig. 6).

Discussion

Activation of $CD4^+$ T cells follows engagement of the TCR by a peptide bound to class II MHC (3), together with a costimulatory signal from the APCs (22). Optimal engagement leads to proliferation, cytokine induction, and effector function. Previous studies have shown that analogues of a native peptide in which a critical TCR binding site was altered may perturb the effector functions of the T cell, including alterations in proliferative capacity and cytokine production (22–25). In this study, we have continued our studies on the design of peptide-based TCR antagonists to reverse the prototypic model of $CD4^+$ T cell–mediated autoimmunity, EAE (9).

We and others (4, 5, 8, 9) have previously formulated nonpathogenic peptide analogues that bind class II MHC to a much greater extent than the native encephalitogenic peptides of MBP and prevent EAE in a specific manner. It has been assumed that the main, if not the only, mechanism by which these peptides inhibit EAE is by successfully competing with the native peptide for binding to MHC class II (26). However, further evidence suggested that mechanisms other than MHC competition might be involved in this process. In the Lewis rat, the major encephalitogenic region encompasses residues 68–86 presented in association with $RT.B^1$ (I-A) MHC class II molecules (20). Arthritogenic T cells recognize their autoantigen in association with $RT.B^1$ gene products as well (27). Wauben et al. (27) have used high MHC-binding peptide analogues of either the encephalitogenic or arthritogenic peptides to show that arthritis could be blocked only by the arthritogenic peptide analogue, whereas EAE could be blocked by either peptide. These findings implied that mechanisms distinct from MHC blocking are operative in this form of immunotherapy (27).

In (PL/JxSJL) mice, EAE is induced by immunization with MBP peptide Ac1–11. Q and K at positions 3 and 4 in this MBP epitope are putative TCR- and MHC-binding sites, respectively (8). Mutations in peptides with either single alanine substitutions at position 4K or double substitutions at both 3Q and 4K resulted in nonencephalitogenic peptides with an enhanced capacity to compete with the native Ac1–11

Table 2. *MBP p87–99[91K>A] Prevents EAE Induced by the Native Peptide in an Epitope-specific Manner**

Group	Immunizing peptides		Peptide 2/ peptide 1 ratio	EAE		
	Peptide 1	Peptide 2		Incidence	Duration	Mean maximum clinical score
					d	
a	p87–99	None		18/18‡	7	2.4 ± 0.2
b	p87–99[91K>A]	None		0/12§	0	0
c	p87–99[95T>A]	None		0/12	0	0
d	p87–99[96P>A]	None		0/12§	0	0
e	p87–99	p87–99	1:1	6/6	8	3 ± 0
f	p87–99	p87–99[95T>A]	1:1	12/12§	7	1.7 ± 0.1
g	p87–99	p87–99[96P>A]	1:1	12/12§	6	2.2 ± 0.2
h	p87–99	p87–99[91K>A]	1:1	0/12§	0	0
i	p87–99	p87–99[91K>A]	1:3	3/6	6	1.2 ± 0.5
j	p68–88	None		6/6	7	3 ± 0
k	p68–88	p87–99[91K>A]	1:1	6/6	7	3 ± 0
l	p1–20	None		0/6	0	0
m	p1–20	p87–99	1:1	6/6	7	2.5 ± 0.2

* Rats were immunized with p87–99/CFA alone or together with different peptide analogues at either a 1:1 or a 1:3 molar ratio. EAE was monitored daily as follows: score 1, paraplegia of the tail; score 2, paralysis of hind limbs; score 3, paralysis of hind and front limbs.
‡ Results of three consecutive experiments done under the same conditions; six rats in each experiment.
§ Results of two consecutive experiments done under the same conditions; six rats in each experiment.

for binding to MHC. Interestingly, only Ac1–11[4K>A], and not the double-substituted analogue Ac1–11[3Q>A, 4K>A] could evoke a proliferative response in encephalitogenic clones selected with the native peptide (5, 8). Only Ac1–11[4A] peptide could inhibit EAE when coadministered in adjuvant with the native peptide (5, 8). These data strongly suggest that factors other than MHC competition are involved, though alternative mechanisms remain elusive. Our results also demonstrate that blockade and reversal of EAE are based on mechanisms that are not explained by MHC competition. T cell unresponsiveness is not seen with the therapeutic peptide analogue used in this study. Of the three peptide analogues, mutated at different TCR interaction sites, only the analogue with the lowest affinity for MHC (p87–99[91K>A]) could prevent actively induced EAE and reverse ongoing disease after adoptive transfer.

De Magistris et al. (19) have demonstrated that peptide analogues, mutated in a TCR putative binding site, may display variable degrees of antagonism toward a collection of T cell clones with similar antigen specificity and MHC restriction (19, 28, 29). A recent paper by Franco et al. (16) examined some peptide analogues, mutated in their TCR binding sites for their potential capability to prevent EAE, when induced by p139–151 of PLP in vivo. No single mutated peptide could antagonize the response of the majority of these PLP clones in vitro. Therefore, they used a pool of TCR antagonists to block the proliferative response of different T cells specific for PLP p139–151. They successfully prevented EAE induction when the antagonists were coadministered in adjuvant at the time of immunization (16). Neither disease reversal nor the use of water soluble inhibitors in vivo was demonstrated.

We tested the therapeutic capabilities of three alanine-substituted analogues, mutated in a putative TCR binding site. All three peptide analogues antagonized the proliferative response of the encephalitogenic line L87–99 (Fig. 4), as well as an anti-p87–99 $CD4^+$ T cell clone selected from this line. Nevertheless, only one of these antagonists could prevent and reverse EAE. In addition, in draining lymph node cells from rats immunized with the inhibitory analogue, there was no reduction in the proliferative response to the pathogenic peptide p87–99 (Fig. 5). This reinforces the idea that EAE resistance does not result from antigen unresponsiveness to p87–99. We hypothesized that the protective and nonprotective antagonists may differentially alter the cytokine profile in the encephalitogenic T cells. Indeed, we have demonstrated that only rats coimmunized with p87–99 and the protective p87–99[91K>A], and not p87–99[95T>A] or 87–99 [96P>A], could downregulate the production of the Th1 cytokines, TNF-α and IFN-γ.

The role of TNF-α in EAE has been studied extensively (30–34). Administration of TNF-α augmented EAE (31), whereas anti-TNF-α therapy abrogated the disease (33, 34). The capacity of autoimmune T cell clones to induce EAE was found to be correlated with their ability to produce TNF-α (32). In different strains of rats susceptibility to EAE was

Table 3. *Treatment of Transferred EAE by Soluble Peptide Analogues*

Group	Soluble peptide	EAE incidence Day 5	Day 6	Day 7	Day 9
	2 mg/ml				
Single inoculation (day 2)*					
a	None	5/5	5/5	5/5	1/5
b	p87–99	0/5	1/5	2/5	0/5
c	p87–99[91K>A]	0/5	1/5	2/5	0/5
e	p87–99[95T>A]	5/5	5/5	5/5	1/5
f	p87–99[96P>A]	3/5	4/5	4/5	0/5
Double inoculation (days 2 and 4)‡					
a	None	4/6	6/6	6/6	1/6
b	p87–99	0/6	0/6	0/6	0/6
c	p87–99[91K>A]	0/6	0/6	0/6	0/6
e	p87–99[95T>A]	2/6	4/6	6/6	1/6
f	p87–99[96P>A]	0/6	1/6	2/6	0/6

* Experiment 1: Rats were inoculated intraperitoneally with 10×10^6 activated L87–99 cells (day 0) and were randomly distributed into five subgroups of five rats each. 2 d later, four of these groups were injected intraperitoneally with either p87–99, p87–99[91K>A], p87–99[95T>A], or p87–99[96P>A]. EAE was monitored daily as described in Table 2.
‡ Experiment 2: Rats were inoculated intraperitoneally with 10×10^6 activated L87–99 cells (day 0) and were randomly distributed into five subgroups of six rats each. Two injections of 2 mg/ml of either p87–99, p87–99[91K>A], p87–99[95T>A], or p87–99[96P>A] were given intraperitoneally on days 2 and 4. EAE was monitored daily as described in Table 2.

correlated with TNF-α expression by astrocytes (30). Elevated levels of TNF-α in the cerebrospinal fluid have been associated with disease progression in MS (35).

The role of IFN-γ in EAE is enigmatic. It is a potent activator of monocytes and macrophages and an inducer of class II expression on astrocytes. Blockade of MHC class II prevents and reverses EAE (36, 37). In EAE, astrocytes may present MBP to encephalitogenic T cells (38). In contrast, intraventricular administration of IFN-γ suppressed EAE in Lewis rats (39), perhaps by eliciting the activation of macrophages, thus resulting in elevated levels of TGF-β secretion, which downregulates TNF-α production and thereby suppresses EAE (40). Anti–IFN-γ antibody also exacerbates EAE (41, 42). Administration of IFN-γ to MS patients induces exacerbations (43).

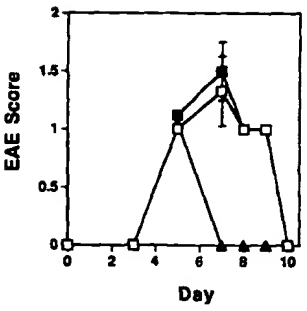

Figure 6. Reversal of EAE by soluble peptide therapy. Rats were immunized with 10^7 L87–99 cells to induce transferred EAE. 5 d later, when clinical disease was apparent, rats were randomly distributed into three subgroups of six rats each. These groups were then injected intraperitoneally with 2 mg/ml of either p87–99-[91K>A] (–▲–), p87–99[95T>A] (–■–), or PBS (–□–). EAE was graded daily (mean ± SE) as described in Table 2.

IFN-γ and TNF-α together exhibit a synergistic effect on enhancing expression of adhesion molecules on endothelial cells (44), and thus act in concert to promote the inflammatory process in the central nervous system. Adhesion molecules such as very late antigen 4 and vascular cell adhesion molecule are critical in the pathogenesis of EAE (45). These roles for TNF-α and IFN-γ may explain why p87–99[91K>A], in contrast to p87–99[95T>A] or p87–99[96P>A] p87, prevented and reversed EAE.

Finally, it is remarkable that the injection of a TCR antagonist in soluble form could reverse ongoing paralysis in EAE. The mechanism of this inhibition is unclear at present, but direct binary engagement of the TCR by antigen may be a possibility, even though this idea is perhaps heretical (46). Binary interactions between superantigen and TCR have been demonstrated (47). Alternatively, soluble MHC–peptide complexes might act to inhibit EAE by engaging TCR without adequate second signals (48).

There is therapeutic potential for the delivery of soluble TCR antagonists devoid of adjuvant or MHC, given the dominance of T cell responses to certain peptides in MS (10, 11). It is noteworthy that the peptide p87–99[91K>A] abrogates the cytotoxicity of a human T cell clone, derived from an MS patient who is HLA DR2, that is cytotoxic for MBP p87–99. This clone has the CDR3 motif LRG in the TCR-β chain (49). Whether suppression of this immune response in an MS patient will alter the course of disease can only be answered in a clinical trial.

It is noteworthy that clinical trials involving the administration of MBP, have been undertaken in patients with MS (50, 51). In one of these trials, some patients became sensitized to the administered MBP (51). Animal testing of native peptides of MBP given subcutaneously in adjuvant (52) or orally (53) has been promising. We propose here that parenteral administration of modified native peptides devoid of encephalitogenic potential that antagonize TCR involved in the disease will be an advantageous strategy for the therapy of MS.

This work was supported by grants from the National Institutes of Health, the National Multiple Sclerosis

Society, the Phil N. Allen Trust, and by gifts from the Mendell family, the Fahnstock family, and Hyman Abadi.

Address correspondence to Dr. N. Karin, Department of Neurology and Neurological Sciences, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, CA 94305-5239.

Received for publication 28 June 1994.

References

1. Ben-Nun, A., H. Wekerle, and I.R. Cohen. 1981. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* 11:195.
2. Karin, N., F. Szafer, D. Mitchell, D.P. Gold, and L. Steinman. 1993. Selective and nonselective stages in homing of T lymphocytes to the central nervous system during experimental allergic encephalomyelitis. *J. Immunol.* 150:4116.
3. Harding, C.V., F. Leyva-Cobian, and E.R. Unanue. 1988. Mechanisms of antigen processing. *Immunol. Rev.* 106:77.
4. Sakai, K., S.S. Zamvil, D.J. Mitchell, S. Hodgkinson, J.B. Rothbard, and L. Steinman. 1989. Prevention of experimental encephalomyelitis with peptides that block interaction of T cells with major histocompatibility complex proteins. *Proc. Natl. Acad. Sci. USA.* 86:9470.
5. Smilek, D.E., D.C. Wraith, S. Hodgkinson, S. Duivedy, L. Steinman, and H.O. McDevitt. 1991. A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA.* 88:9633.
6. Steinman, L. 1991. The development of rational strategies for selective immunotherapy against demyelinating disease. *Adv. Immunol.* 49:357.
7. Wauben, M.H., I. Joosten, A. Schlief, R. van der Zee, C.J. Boog, and E.W. van Eden.1994. Inhibition of experimental autoimmune encephalomyelitis by MHC class II binding competitor peptides depends on the relative MHC binding affinity of the disease-inducing peptide. *J. Immunol.* 152:4211.
8. Wraith, D.C., D.E. Smilek, D.J. Mitchell, L. Steinman, and H.O. McDevitt. 1989. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell.* 59:247.
9. Wraith, D.C., H.O. McDevitt, L. Steinman, and H. Acha-Orbea. 1989. T cell recognition as the target for immune intervention in autoimmune disease. *Cell.* 57:709.
10. Oksenberg, J.R., M.A. Panzara, A.B. Begovich, D. Mitchell, H.A. Erlich, R.S. Murray, R. Shimonkevitz, M. Sherritt, J. Rothbard, C.C. Bernard, and L. Steinman. 1993. Selection for T cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature (Lond.).* 362:68.
11. Gold, D.P., M. Vainiene, B. Celnik, S. Wiley, C. Gibbs, G.A. Hashim, A.A. Vandenbark, and H. Offner. 1992. Characterization of the immune response to a secondary encephalitogenic epitope of basic protein in Lewis rats. II. Biased T cell receptor V beta expression predominates in spinal cord infiltrating T cells. *J. Immunol.* 148:1712.
12. Wilson, D.B., L. Steinman, and D.P. Gold. 1993. The V-region disease hypothesis: new evidence suggests it is probably wrong. *Immunol. Today.* 14:376.
13. Steinman, L. 1994. Specific motifs in T cell receptor VβDβJβ gene sequences in multiple sclerosis lesions. *Behring. Inst. Mitt.* 94:148.
14. Martin, R., D. Jaraquemada, M. Flerlage, J. Richert, J. Whitaker, E.O. Long, D.E. McFarlin, and H.F. McFarland. 1990. Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* 145:540.
15. Ota, K., M. Matsui, E.L. Milford, G.A. Mackin, H.L. Weiner, and D.A. Hafler. 1990. T cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature (Lond.).* 346:183.
16. Franco, A., S. Southwood, T. Arrhenius, H.M. Grey, A. Sette, and G.Y. Ishioka. 1994. T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis. *Eur. J. Immunol.* 24:940.
17. Mozes, E., M. Dayan, E. Zisman, S. Brocke, A. Licht, and I. Pecht. 1989. Direct binding of a myasthenia gravis related epitope to MHC class II molecules on living murine antigen presenting cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4049.
18. Gautam, A., C. Lock, D. Smilek, C. Pearson, L. Steinman, and H.O. McDevitt. 1994. Minimum structural requirements for peptide presentation by major histocompatibility complex class II molecules: Implications in induction of autoimmunity. *Proc. Natl. Acad. Sci. USA.* 91:767.
19. De Magistris, M.T., J. Alexander, M. Coggeshall, A. Altman, F.C. Gaeta, H.M. Grey, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell.* 68:625.
20. Offner, H., G.A. Hashim, B. Celnik, A. Galang, X.B. Li, F.R. Burns, N. Shen, K.E. Heber-Katz, and A.A. Vandenbark. 1989. T cell determinants of myelin basic protein include a unique encephalitogenic I-E-restricted epitope for Lewis rats. *J. Exp. Med.* 170:355.
21. Offner, H., M. Vainiene, D.P. Gold, B. Celnik, R. Wang, G.A. Hashim, and A.A. Vandenbark. 1992. Characterization of the immune response to a secondary encephalitogenic epitope of basic protein in Lewis rats. I. T cell receptor peptide regulation of T cell clones expressing cross-reactive V beta genes. *J. Immunol.* 148:1706.
22. Muller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus clonal inactivation: a costimulatory signaling pathway determines the outcome of TCR occupancy. *Annu. Rev. Immunol.* 7:445.
23. Evavold, B.D., and P.M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science (Wash. DC).* 252:1308.
24. Evavold, B.D., L.J. Sloan, B.L. Hsu, and P.M. Allen. 1993. Separation of T helper 1 clone cytolysis from proliferation and lymphokine production using analog peptides. *J. Immunol.* 150:3131.
25. Sloan, L.J., B.D. Evavold, and P.M. Allen. 1993. Induction

of T cell anergy by altered T cell-receptor ligand on live antigen-presenting cells. *Nature (Lond.).* 363:156.
26. Gautam, A.M., C.I. Pearson, A.A. Sinha, D.E. Smilek, L. Steinman, and H.O. McDevitt. 1992. Inhibition of experimental autoimmune encephalomyelitis by a nonimmunogenic non-self peptide that binds to I-Au. *J. Immunol.* 148:3049.
27. Wauben, M.H., C.J. Boog, R. van der Zee, I. Joosten, A. Schlief, and W. van-Eden. 1992. Disease inhibition by major histocompatibility complex binding peptide analogues of disease-associated epitopes: more than blocking alone. *J. Exp. Med.* 176:667.
28. Ostrov, D., J. Krieger, J. Sidney, A. Sette, and P. Concannon. 1993. T cell receptor antagonism mediated by interaction between T cell receptor junctional residues and peptide antigen analogues. *J. Immunol.* 150:4277.
29. Ruppert, J., J. Alexander, K. Snoke, M. Coggeshall, E. Herbert, D. McKenzie, H.M. Grey, and A. Sette. 1993. Effect of T cell receptor antagonism on interaction between T cells and antigen-presenting cells and on T cell signaling events. *Proc. Natl. Acad. Sci. USA.* 90:2671.
30. Chung, I.Y., J.G. Norris, and E.N. Benveniste. 1991. Differential tumor necrosis factor alpha expression by astrocytes from experimental allergic encephalomyelitis–susceptible and –resistant rat strains. *J. Exp. Med.* 173:801.
31. Kuroda, Y., and Y. Shimamoto. 1991. Human tumor necrosis factor-alpha augments experimental allergic encephalomyelitis in rats. *J. Neuroimmunol.* 34:159.
32. Powell, M.B., D. Mitchell, J. Lederman, J. Buckmeier, S.S. Zamvil, M. Graham, N.H. Ruddle, and L. Steinman. 1990. Lymphotoxin and tumor necrosis factor-alpha production by myelin basic protein–specific T cell clones correlates with encephalitogenicity. *Int. Immunol.* 2:539.
33. Ruddle, N.H., C.M. Bergman, K.M. McGrath, E.G. Lingenheld, M.L. Grunnet, S.J. Padula, and R.B. Clark. 1990. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J. Exp. Med.* 172:1193.
34. Selmaj, K., C.S. Raine, and A.H. Cross. 1991. Anti-tumor necrosis factor therapy abrogates autoimmune demyelination. *Ann. Neurol.* 30:694.
35. Sharief, M.K., and R. Hentges. 1991. Association between TNFα and disease progression in patients with MS. *N. Engl. J. Med.* 325:467.
36. Sriram, S., and L. Steinman. 1983. Anti I-A antibody suppresses active encephalomyelitis: treatment model for diseases linked to IR genes. *J. Exp. Med.* 158:1362.
37. Steinman, L., J.T. Rosenbaum, S. Sriram, and H.O. McDevitt. 1981. In vivo effects of antibodies to immune response gene products: prevention of experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA.* 78:7111.
38. Fontana, A., W. Fierz, and H. Wekerle. 1984. Astrocytes present myelin basic protein to encephalitogenic T cell line. *Nature (Lond.).* 307:273.
39. Voorthuis, J.A., B.M. Uitdehaag, C.J. De Groot, P.H. Goede, P.H. van der Meide, and C.D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin. Exp. Immunol.* 81:183.
40. Santambrogio, L., G.M. Hochwald, B. Saxena, C.H. Leu, J.E. Martz, J.A. Carlino, N.H. Ruddle, M.A. Palladino, L.I. Gold, and G.J. Thorbecke. 1993. Studies on the mechanisms by which transforming growth factor-beta (TGF-beta) protects against allergic encephalomyelitis. Antagonism between TGF-beta and tumor necrosis factor. *J. Immunol.* 151:1116.
41. Billiau, A., H. Heremans, F. Vandekerckhove, R. Dijkmans, H. Sobis, E. Meulepas, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J. Immunol.* 140:1506.
42. Duong, T.T., J. St. Louis, J.J. Gilbert, F.D. Finkelman, and G.H. Strejan. 1992. Effect of anti-interferon-gamma and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. *J. Neuroimmunol.* 36:105.
43. Panitch, H.S., R.I. Heracl, J. Schindler, and K.P. Johnson. 1987. Treatment of MS with gamma interferon: exacerbations associated with activation of the immune system. *Neurology.* 37:1097.
44. Thornbill, M.H., S.M. Wellicome, D.L. Mahiouz, J.S. Lanchbury, A.U. Kyan, and D.O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-γ to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J. Immunol.* 146:592.
45. Yednock, T.A., C. Cannon, L.C. Fritz, M.F. Sanchez, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature (Lond.).* 356:63.
46. Steinman, L., I. Cohen, D. Teitelbaum, and R. Arnon. 1977. Regulation of autosensitization to encephalitogenic myelin basic protein by macrophage-associated and soluble antigen. *Nature (Lond.).* 265:173.
47. Seth, A., L. Stern, T. Ottenhoff, I. Engel, M. Owen, J.R. Lamb, R.D. Klausner, and D.C. Wiley. 1994. Binary and ternary complexes between T cell receptor, class II MHC and superantigen in vitro. *Nature (Lond.).* 369:324.
48. Sharma, S.D., B. Nag, X.M. Su, D. Green, E. Spack, B.R. Clark, and S. Sriram. 1991. Antigen-specific therapy of experimental allergic encephalomyelitis by soluble class II major histocompatibility complex–peptide complexes. *Proc. Natl. Acad. Sci. USA.* 88:11465.
49. Martin, R., U. Utz, J.E. Coligan, J.R. Richert, M. Flerlage, E. Robinson, R. Stone, W.E. Biddison, D.E. McFarlin, and H.F. McFarland. 1992. Diversity in fine specificity and T cell receptor usage of the human $CD4^+$ cytotoxic T cell response specific for the immunodominant myelin basic protein peptide 87-106. *J. Immunol.* 148:1359.
50. Campbell, B., P. Vogel, E. Fisher, and R. Lorenz. 1973. Myelin basic protein administration in MS. *Arch. Neurol.* 29:10.
51. Salk, J., F.C. Westall, J.S. Romine, and W. Wiederholt. 1980. Studies on myelin basic protein administration in MS patients. *In* Progress in MS Research. H.J. Bauer, S. Pser, and G. Ritter, editors. Springer-Verlag, Berlin. pp. 419–435.
52. Gaur, A., W. Brook, A. Liu, J. Rothbard, and G. Fathman. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science (Wash. DC).* 258:1491.
53. Miller, A., O. Lider, and H.L. Weiner. 1991. Antigen-driven bystander suppression following oral administration of antigens. *J. Exp. Med.* 174:791.